OIPE JC106 NOV 22 2004 PATENT & TRADEMARK OFFICE

MAIL STOP
AMENDMENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	C.A. Blau et al.	Attorney Docket No.:	UWOTL115624
Application No.:	09/582,916	Group Art Unit:	1632
Filed:	October 2, 2000	Examiner:	A.M.S. Wehbe

Title: METHODS OF CONTROLLING CELL DIFFERENTIATION AND GROWTH USING A FUSION PROTEIN AND A DRUG

INVENTOR'S DECLARATION UNDER 37 C.F.R. § 1.132

Seattle, Washington 98101

November 17, 2004

TO THE COMMISSIONER FOR PATENTS:

I, Dr. Carl Anthony Blau, declare as follows:

1. I am a co-inventor named in the above-identified patent application and I am familiar with the subject matter of this application.

2. My educational and work background are as follows: I received a Bachelor of Science degree from Wright State University in Dayton, Ohio, in 1982, and a Doctor of Medicine degree from Ohio State University in Columbus, Ohio, in 1986. I was an Intern in the Department of Medicine at Duke University in Durham, North Carolina, from 1986 to 1987, and a Resident in the Department of Medicine at Duke University in Durham, North Carolina, from 1987 to 1989. From 1989 to 1994, I was a Senior Fellow in the Division of Oncology at the University of Washington in Seattle, Washington. I am currently an Associate Professor in the Division of Hematology of the Department of Medicine and an Adjunct Associate Professor of Genome Sciences at the University of Washington, Seattle, and a Member of the Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium. I have extensive experience in gene transfer to and expansion of primary hematopoietic cells, such as hematopoietic stem cells.

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3. It is my understanding that U.S. Patent No. 5,741,899 (Capon et al.) has been cited as a prior art reference in the above-identified application.

4. The methods and cells claimed in this application, as amended, are directed to primary mammalian cells—such as hematopoietic stem cells—containing a construct encoding a fusion protein comprising at least one signaling domain and at least one drug-binding domain, wherein exposure to a drug of primary cells transduced with this construct reversibly induces growth, proliferation, or differentiation of the cells. Capon et al. does not provide an enabling description that would permit one skilled in the art to make and use primary mammalian cells (such as primary hematopoietic stem cells) containing a construct encoding a fusion protein comprising at least one signaling domain and at least one drug-binding domain.

5. Capon et al. does not provide an enabling description of methods of expanding primary mammalian cells (for example, primary hematopoietic stem cells) or methods of treating a hematopoietic disease or condition by exposing cells containing a construct coding for a fusion protein comprising at least one signaling domain and at least one drug-binding domain to the drug. Capon et al. describes placing CPR-expressing $CD8^+$ T cells in "culture dishes coated with saturating concentrations of either OCTK4A, anti-human Fc Mab, gp120, gp160-expressing cells, gp41/gp120-expressing cells, HIV-1 infected cells or FK1012" (Col. 42, lines 61-64). However, the use of "saturating concentrations" of FK1012 would not result in proliferation because saturating concentrations of FK1012 inhibit growth by occupying all of the FKBP sites and thereby prevent dimerization, as further described below.

6. FK1012 is a bivalent drug that induces dimerization of two FKBP domains. It has been shown that high concentrations of a bivalent drug prevent dimerization of a receptor for that drug. For example, human growth hormone (hGH) is a bivalent molecule with two separate sites for binding to the extracellular domain of the human growth hormone receptor (hGHbp) (Fuh et

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al. (1992) *Science* 256:1677-80, page 1678, Col. 1; Fig. 1; enclosed). At low concentrations, hGH binds to both sites to produce an active complex containing a dimeric form of hGHbp $(hGHpb)_2$, thereby inducing proliferation (Fuh et al. (1992) *Science* 256:1677-80, page 1678, Col. 1; Fig. 1). However, excess hGH will dissociate this dimeric complex and inhibit proliferation (Fuh et al. (1992) *Science* 256:1677-80, page 1678, Col. 1-2; Fig.1). Accordingly, at high concentrations, human growth hormone saturates the receptor and acts as an antagonist by preventing dimerization (Fuh et al. (1992) *Science* 256:1677-80, page 1678, Col. 2; Fig. 1).

7. The same effect is observed with saturating concentrations of FK1012. Proliferation of the murine cell line Ba/F3 is dependent on the presence cytokine IL-3. However, Ba/F3 cells expressing a chimeric protein containing an intracellular signaling domain linked to a FKBP domain are capable of FK1012-dependent proliferation in the absence of IL-3 (see, e.g., Blau et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:3076-81, abstract; of record). Proliferation of these cells in response to FK1012 is concentration-dependent, and at higher concentrations of FK1012 less proliferation is observed (Blau et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:3076-81, page 3078, Col. 1). The reason for the decline in cell proliferation with higher concentrations of FK1012 is suggested to be "due to excessive occupancy of the FKBP12 binding sites by FK1012, thus preventing oligomerization/dimerization of the fusion proteins" (Blau et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:3076-81, page 3078, Col. 1). These results indicate that saturating concentrations of FK1012 would inhibit proliferation. Confirmation of this theoretical certainty is provided by the following experimental results obtained in my laboratory on June 2, 1997, showing that saturating concentrations of FK1012 inhibit proliferation of cells expressing a similar chimeric protein. A clone of Ba/F3 cells expressing a chimeric protein containing an FKBP domain linked to the Flt intracellular signaling domain (F1Flt3) was exposed to increasing concentrations of FK1012 and cell proliferation was

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measured using a previously described assay (Blau et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:3076-81, page 3077, Col. 1-2). The amount of cell proliferation was compared to the proliferation obtained in the presence of IL-3-containing medium (WEHI-conditioned medium). As shown in Table 1 and Figure 1, FlFlt3-containing BA/F3 cells are capable of FK1012-dependent proliferation in the absence of IL-3. The maximum amount of proliferation of this clone of F1Flt3-expressing cells was obtained at 1 nM of FK1012. However, concentrations of FK1012 above 10 nM were found to inhibit cell proliferation and at concentrations above about 100 nM FK1012, no cell growth was observed (Table 1, Figure 1). These results unequivocally demonstrate that concentrations of FK1012 sufficient to saturate all of the FKBP sites completely prevent FK1012-induced cell proliferation, similar to the results described for hGH. Because the mechanisms of dimerization of a receptor for a bivalent drug is the same in primary cells, saturating concentrations of a bivalent drug would also prevent drug-induced proliferation of primary mammalian cells.

Table 1. Effect of Increasing Concentrations of FK1012 on Cell Proliferation in F1Flt3-Containing Ba/F3 Cells

Concentration of FK1012 (nM)	**Growth Relative to 1% WEHI**
0	0
0.01	0.087065
0.1	0.639303
1	2.281095
10	0.455224
100	0.062189
1000	-0.012438

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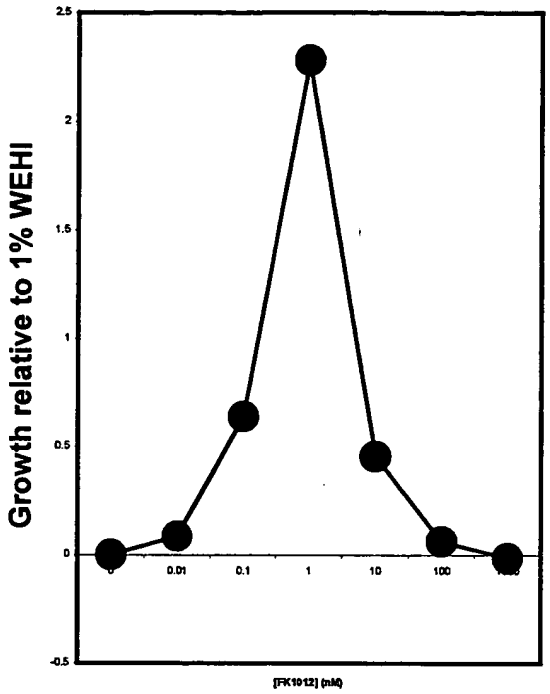
FIGURE 1.



8. Because Capon et al. teaches the use of saturating concentrations of FK1012, a person of skill in the art reading Capon et al. would not be able to (1) obtain, without undue experimentation, a genetically engineered primary mammalian cell containing a construct coding for a fusion protein comprising at least one signaling domain and at least one drug-binding domain to a drug, wherein administration of the drug to the cell induces growth, proliferation and/or differentiation, or (2) practice, without undue experimentation, methods of expanding primary mammalian cells—including hematopoietic stem cells—or methods of treating a hematopoietic disease or condition by exposing cells containing a construct coding for a fusion

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protein comprising at least one signaling domain and at least one drug-binding domain to a drug, as described and claimed in the above-identified patent application.

9. All statements made herein and of my own knowledge are true, and all statements made on information and belief are believed to be true; and further, these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,

Dated: 11/17/04

C. A. Blau

Dr. C.A. Blau

KBB:kbb

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CURRICULUM VITAE

Carl Anthony Blau

PERSONAL DATA

Date of Birth: 7/28/60

Place of Birth: Clinton, Indiana

EDUCATION

M.D., Ohio State University, 1986, Cum Laude

B.S., Wright State University, 1982, Summa Cum Laude

POSTGRADUATE TRAINING

1989 - 1994	Senior Fellow, Division of Oncology, University of Washington
1987-1989	Resident, Department of Medicine, Duke University
1986-1987	Intern, Department of Medicine, Duke University

FACULTY POSITIONS HELD

2003-present	Member, Fred Hutchinson/University of Washington Cancer Consortium
2003-present	Appointments and Promotions Committee, Department of Medicine, University of Washington
2003-present	Adjunct Associate Professor of Genome Sciences, University of Washington
2000-present	Associate Professor, Division of Hematology, Department of Medicine, University of Washington
1998-2004	Associate Program Director for Gene and Cell Therapy, General Clinical Research Center, University of Washington
1997-2000	Assistant Professor, Division of Hematology, Department of Medicine, University of Washington, Seattle, Washington
1994-1997	Acting Assistant Professor, Division of Hematology, Department of Medicine, University of Washington, Seattle, Washington

HOSPITAL POSITIONS HELD

Scientific Advisory Board, General Clinical Research Center, University of Washington, Seattle, WA 7/95 - present.

HONORS AND AWARDS

American Society of Clinical Investigation, 2002

American Society of Hematology Junior Faculty Scholar Award, 1997.

Clinical Associate Physician Award, Clinical Research Center, 1994-1997

National Research Scientist Award, 1992-1994.

Cooley's Anemia Foundation Award, 1992-1993.

Haskill Schiff Award, Duke University Department of Medicine, 1989 (awarded to one resident annually for excellence in clinical medicine).

Alpha Omega Alpha, 1985.

Rotary Club Exchange Student to Ravensburg, Germany, July 1977 – June 1978.

PRESENTATIONS AT NATIONAL AND INTERNATIONAL MEETINGS

Invited Speaker, "

Invited Speaker, "Modern Trends in Human Leukemia" XV Wilsede Meeting, Wilsede Germany, June 14-18, 2003. (Robert Richard sent in my place).

Invited Speaker, Thirteenth Conference on Hemoglobin Switching, Oxford, UK September 25-29, 2002.

Invited Speaker, Scientific Symposium, Hemopoietic Gene Therapy: Progress and Prospects, American Society of Gene Therapy, Boston, MA, June 7, 2002.

Invited Speaker, Third Conference on Stem Cell Gene Therapy, Biology and Technology, Rockville, Maryland, March 22, 2002

Invited Speaker, Eastlick Symposium, Washington State University, March 2001.

Invited Speaker, Hemoglobin Switching Conference, Orcas Island, 2000.

Invited Speaker, "Modern Trends in Leukemia," Wilsede, Germany, June 2000.

Meet the Investigator Session, American Society of Gene Therapy, Denver, June 2000.

Invited Speaker, Baylor College of Medicine, March 2000.

Invited Speaker, University of Pittsburgh Medical Center Hematology/Oncology Grand Rounds, February, 2000.

Invited Speaker, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, October 8, 1999.

Invited Speaker, Fourth Symposium of Tissue Engineering for Therapeutic Use, Kyoto, Japan, September 1999.

Invited Speaker, University of Alabama, Birmingham, July 1999.

New Investigator Plenary Session, International Society for Experimental Hematology, Monte Carlo, Monaco, June 1999.

Invited Speaker, Albert Einstein College of Medicine, Bronx, N.Y., June 1999.

Department of Medicine Grand Rounds, University of Washington, April 29, 1999.

Science in Medicine Lecturer, University of Washington, November 1998.

New Investigator Plenary Session, International Society for Experimental Hematology, Vancouver, Canada, August 1998.

Invited Speaker, Japanese Biochemical Society's Conference on Stem Cell Commitment and Expansion, Tsukuba, Japan, July 8-10, 1998.

Invited Speaker, Second Conference on Stem Cell Gene Therapy, Orcas Island, WA, June 1998.

Plenary Session: American Society of Gene Therapy, Seattle, WA, May 1998.

Invited Speaker, Keystone Conference on Stem Cell Gene Therapy , Lake Tahoe, NV, February, 1998.

Conference on In Utero Transplantation and Stem Cell Gene Therapy, 1996

Hemoglobin Switching Conference, Orcas Island, 1996

First Conference on Stem Cell Gene Therapy, 1995

Hemoglobin Switching Conference, Orcas Island, 1994

American Society of Hematology, 1994

NATIONAL RESPONSIBILITIES

Panelist, Comprehensive Sickle Cell Centers – Working Group Meeting, May 15, 2000.

Member, Special Study Section for Tissue Engineering, July 5 2000.

Member, Special Study Section for Tissue Engineering, March 24, 2001.

External Referee for the Medical Research Council's Molecular Hematology Unit, Oxford, England, April 2001.

External Referee for Swiss National Science Foundation,April 2001.

Member, Special Study Section, National Center for Research Resources, General Clinical Research Center, USC, Los Angeles, February, 2002.

Member, Special Emphasis Panel for the National Sickle Cell Centers, May 6 – 8, 2002.

Member, External Advisory Board, Program Project Grant: "Gene Therapy Using Hematopoietic Stem Cells." 1P01HL073104-01, Principal investigator: Don Kohn, USC, 2002 – present.

Member, Scientific Committee on Hemopoietic Cell Gene Therapy, American Society of Gene Therapy, 1999 – 2002.

BOARD CERTIFICATIONS

National Medical Boards, parts 1-3
American Board of Internal Medicine, 1989
American Board of Internal Medicine, Medical Oncology, 1991
American Board of Internal Medicine, Hematology, 1992
Certificate of Completion, Clinical Gene Transfer Training Course May 29-30, 2001.

RESEARCH SUPPORT

A. Active Support

R01 DK52997 (Blau) 8/10/97 – 7/31/05 15%
NIH/NIDDK $179,000 (annual direct support)
A Proliferative Switch for Genetically Modified Cells
Specific Aim 1 will humanize the mpl vector. Specific aim 2 will test the humanized vector in normal mice and in a mouse model of mpl deficiency. Specific aim 3 will test the humanized vector in a canine model. Specific aim 4 will test the humanized vector in hemopoietic cells taken from patients with mpl deficiency.
No overlap.

R01 DK57525-02 (Blau) 9/1/99-8/31/03 (no cost extension) 15%
NIH/NIDDK $250,000 (annual direct support)
Mixed Chimerism in the Hemoglobinopathies
Specific Aim 1 tests whether CIDs can expand genetically modified stem and progenitor cells in vivo. Specific Aim 2 develops a mouse model of mixed chimerism in beta thalassemia, and seeks to determine the level of normal donor stem cell engraftment needed to reverse the thalassemic phenotype. Specific Aim 3 tests whether CID-mediated in vivo expansion of normal donor stem cells can correct the thalassemic phenotype of mice with mixed chimerism. Specific Aim 4 evaluates whether FL can sensitize stem ells to 5-FU. In Specific Aim 5, the FL/5-FU combination is added to an immunosuppressive condition regimen for studies in allogeneic models of mixed chimerism. In Specific Aim 6, findings from the previous specific aims are combined to test CID-mediated in vivo expansion of normal donor stem cells in an allogeneic model of mixed chimerism. Specific Aim 1 overlaps with Specific Aim 1 of P01HL53750.

R01 DK61844-01 (Blau) 9/1/01-8/31/05 20%
NIH/NIDDK $225,000 (annual direct support)
Hemopoietic Stem Cell Plasticity
Here we propose to characterize the developmental potential of human hemopoietic cells, and to develop a method that will allow for the pharmacologically controlled *in situ* expansion of cells that have transited from hemopoietic to non-hemopoietic tissues, using liver as a model. Our approach involves expressing a protein that induces cell growth in the presence of a chemical dimerizing agent. In Specific Aim 1 we will test the developmental potential of human hemopoietic cells. We will study autopsy tissues taken from female recipients of male bone marrow cells to determine whether male cells contribute to various non-hemopoietic tissues. In Specific Aim 2 we will test whether genetically modified hemopoietic cells retain hepatocyte potential. Bone marrow from male mice will be transplanted into female recipients who will then be analyzed for hepatocytes arising from the

male donor. In Specific Aim 3 we will construct and test vectors for expanding marrow derived hepatocytes. In Specific Aim 4 we will use chemical dimerizing agents to expand marrow-derived hepatocytes, *in vivo*.

P01 HL53750-02 (Stamatoyannopoulos) 9/01/00-8/31/04 10%
NIH/NHLBI $169,360 (annual direct support)
Subproject 4: Stem Cell Expansion Using Chemical Inducers of Dimerization (Blau).
The specific aims of the project are: 1) to test whether CID-mediated activation of mpl allows for the in vivo selection of transduced murine stem and progenitor cells; 2) to identify and eliminate mpl maturational signaling domains to produce a derivative that is capable of proliferative signaling but incapable of maturational signaling; 3) to test whether CID-mediated activation of mpl allows for the selection and expansion of transduced human CD34+ cells in vitro and in vivo; 4) to test in vivo selection in a large animal model using CIDs; 5) to test vectors that contain both a CID-selectable gene and a γ globin gene for studies of selection in normal mice and in a mouse model of sickle cell anemia. No overlap.

P01 DK55820-01-A1 (Stamatoyannopoulos) 5/01/00-4/30/05 10%
NIH/NIDDK $104, 519 (annual direct support)
Subproject 2: Receptor Specificity in the Proliferation and Differentiation of Hemopoietic Stem Cells (Blau)
Studies described in this proposal will identify and characterize the features of mpl signaling that are permissive for stem cell expansion. Experiments will be performed to determine whether mpl's ability to stimulate stem cell expansion is shared by the GCSF receptor. Finally, transgenic mice will be used to quantitate the magnitude and kinetics of stem cell expansion occurring in response to mpl and GCSF receptor signaling. Studies will be performed to determine whether transient activation of the mpl and GCSF receptor can influence the developmental fate of stem cells. No overlap.

U01 HL66947-01 (Stamatoyannopoulos) 9/28/00-8/31/05 5%
NIH/NHLBI $181,953 (annual direct support)
The UW/FHCRC Program of Excellence in Gene Therapy, Core A: Clinical Core (Blau)
The goal of this program is to combine the resources of two institutions, the University of Washington and the Fred Hutchinson Cancer Research Center, to advance gene therapy in two areas of interest to NHLBI- stem cell gene therapy of hematological disorders and gene therapy of two common inherited lung diseases. No overlap.

M01 RR00037-41 (Ramsey) 12/1/02-3/31/08 20%
NIH/NCRR $6,433,420 (annual direct support for GCRC)
General Clinical Research Center: Associate Program Director for Gene and Cell Therapy (Blau)
The goal of the General Clinical Research Center is to provide research infrastructure to the faculty members at the University of Washington, Children's Hospital & Regional Medical Center, Fred Hutchinson Cancer Research Center, the Seattle VA and Harborview Medical Center. No overlap.

1P20 GM69983-01 (Blau) 9/30/03-8/31/06 15%
NIH/NIGMS $520,338 (annual direct support)
UW/FHCRC Exploratory Center for Human ES Cell Research (Blau) $500,000/yr (requested)
This application proposes to build an infrastructure for human embryonic stem cell research that will serve both the University of Washington and the Fred Hutchinson Cancer Research Center.

Fundamental to this effort will be the establishment of a Human Embryonic Stem Core Laboratory, which will provide a centralized facility for the culture, maintenance, manipulation, and differentiation of human ES cells, and will serve as a resource for support and training of the local research community. Three Pilot Projects will complement the Core Laboratory to establish a foundation for human ES cell research in Seattle

PATENTS

USPTO Application No. 09/582,916 filed October 2, 2000 Entitled: "Methods of Controlling Cell Differentiation and Growth using a Fusion Protein and a Drug" UWOTL 2211-2733-3471PT Inventors: C. Anthony Blau and David Spencer.

LICENSE

Washington State Medical License number 0026522; 1989 - present

PROFESSIONAL ORGANIZATIONS

American Society of Clinical Investigation
American Society of Hematology
American Society of Gene Therapy

BIBLIOGRAPHY

Publications

1. Abkowitz JL, Broudy VC, Sabo KM, Nakamoto B, Blau CA, Martin FH, Zsebo KM, and Papayannopoulou Th: Diamond Blackfan anemia: In vitro response of erythroid progenitors to c-kit ligand. **Blood** 78:2198-2202, 1991.

2. Blau CA, Constantoulakis P, Al-Khatti A, Spadaccino E, Goldwasser E, Papayannopoulou Th, and Stamatoyannopoulos G: Fetal hemoglobin in acute and chronic states of erythroid expansion. **Blood** 81:227-233,1993.

3. Blau CA, Constantoulakis P, Shaw CM, and Stamatoyannopoulos G: Fetal hemoglobin induction with butyric acid: Efficacy and toxicity. **Blood** 81:529-537,1993.

4. Papayannopoulou T, Brice M, and Blau CA: Kit ligand in synergy with interleukin-3 amplifies the erythropoietin-independent, globin-synthesizing progeny of normal human burst-forming units-erythroid in suspension cultures: physiologic implications. **Blood** 81:299-310,1993.

5. Palena A, Blau A, Stamatoyannopoulos G, and Anagnou NP: Molecular characterization of a novel ($\delta\beta$)-thalassemia deletion with increased expression of fetal hemoglobin in a family of Eastern European origin. **Blood** 83:3738-3745, 1994.

6. Stamatoyannopoulos G, Blau CA, Nakamoto B, Josephson B, Li Q, Liakopoulou E, Pace B, Papayannopoulou Th, Brusilow S, and Dover G: Fetal hemoglobin induction by acetate, a product of butyrate catabolism. **Blood** 84:3198-3204, 1994.

7. Liakopoulou E, Blau CA, Li Q, Josephson G, Wolf JA, Fournarakis B, Raisys V, Dover G, Papayannopoulou Th, and Stamatoyannopoulos G: Stimulation of fetal hemoglobin production by short chain fatty acids. **Blood** 86:3227-3235, 1995.

8. Neff T and Blau CA: Forced expression of cytidine deaminase confers resistance to cytosine arabinoside and gemcitabine. **Experimental Hematology** 24:1340-1346, 1996.

9. Blau CA, Neff T and Papayannopoulou Th: The hematological effects of folate analogs: Implications for using the dihydrofolate reductase gene for in vivo selection. **Human Gene Therapy** 7: 2069-2078, 1996.

10. Blau CA, Neff T and Papayannopoulou Th: Cytokine prestimulation as a gene therapy strategy: Implications for using the MDR1 gene as a dominant selectable marker. **Blood** 89:146-154, 1997.

*11. Blau CA, Peterson KR, Drachman JG and Spencer DM: A proliferation switch for genetically modified cells. **Proceedings of the National Academy of Sciences USA** 94:3076-3081, 1997.

12. Jin L, Asano H and Blau CA: Stimulating cell proliferation through the pharmacologic activation of c-*kit*. **Blood** 91:890-897, 1998.

13. Abboud M, Laver J and Blau CA: Granulocytosis causing sickle-cell crisis. **Lancet** 351:609, 1998.

14. Li Q, Blau CA, Clegg CH, Rohde A, Stamatoyannopoulos G: Multiple epsilon promoter elements participate in the developmental control of the epsilon globin genes in transgenic mice. **Journal of Biological Chemistry** 273:17361-17367, 1998.

15. Jin L, Siritanaraktul N, Emery DW, Richard RE, Kaushansky K, Papayannopoulou Th and Blau CA: Targeted expansion of genetically modified bone marrow cells. **Proceedings of the National Academy of Sciences USA** 95:8093-8097, 1998.

16. Jin L, Neff T and Blau CA: Marrow sensitization to 5 fluorouracil using the ligands for flt-3 and c-*kit*. **Experimental Hematology** 27:520-525, 1999.

17. Richard RE, Wood B, Zeng H, Papayannopoulou Th and Blau CA: Expansion of genetically modified primary human hemopoietic cells using chemical inducers of dimerization. **Blood** 95:430-6, 2000.

*18. Jin L, Zeng H, Otto KG, Richard RE, Emery DW and Blau CA: In vivo selection using a cell growth switch. **Nature Genetics**, 26:64-66, 2000.

19. Otto KG, Jin L, Spencer DM and Blau CA: Cell Proliferation Induced by Forced Engagement of c-Kit and Flt-3. **Blood** 97:3662-3664, 2001.

*20. Zeng H, Masuko M, Jin L, Neff T, Otto KG and Blau CA: Receptor specificity in the self-renewal and differentiation of primary multipotential hemopoietic cells. **Blood** 98:328-334, 2001.

21. Otto KG, Broudy VC, Lin N, Parganas E, Drachman JG, Luthi JN, Ihle JN and Blau CA: Membrane localization is not required for mpl function in normal hematopoietic cells. **Blood** 98:2077-2083, 2001.

22. Whitney ML, Otto K, Blau CA, Reinecke H and Murry CE: Control of myoblast proliferation with a synthetic ligand. **Journal of Biological Chemistry** 276:41191-41196, 2001.

23. Ieremia J and Blau CA: Limitations of a mouse model of sickle cell anemia. **Blood Cells, Molecules & Diseases** 28:146-151, 2002.

24. Li Z-Y, Otto KG, Richard RE, Ni S, Kirillova, I, Fausto, N, Blau CA and Lieber, A: Dimerizer-induced proliferation of genetically modified hepatocytes. **Molecular Therapy** 5:420-6, 2002.

*25. Zhao S, Zoller K, Masuko M, Rojnuckarin P, Yang X, Parganas E, Kaushansky K, Ihle JN, Papayannopoulou Th, Willerford DM, Clackson T and Blau CA: JAK2, complemented by a second signal from c-kit or flt-3, triggers extensive self-renewal of primary multipotential hemopoietic cells. **EMBO Journal** 21:2159-2167, 2002.

*26. Neff T, Horn PA, Valli VE, Gown AM, Wardwell S, Wood BL, von Kalle C, Schmidt M, Peterson LJ, Morris JC, Richard RE, Clackson T, Kiem HP, Blau CA: Pharmacologically regulated in vivo selection in a large animal. **Blood** 100: 2026-2031, 2002.

27. Berger C, Blau CA, Clackson T, Riddell SR, Heimfeld S: CD28 costimulation and immunoaffinity-based selection efficiently generate primary gene-modified T cells for adoptive immunotherapy. **Blood** 101: 476-484, 2003.

28. Richard RE and Blau CA: Selective expansion of genetically modified cord blood cells. **Stem Cells** 21: 71-78, 2003.

29. Berger C, Blau CA, Huang M-L, Iuliucci JD, Dalgarno DC, Gaschet J, Heimfeld S, Clackson T, Riddell SR: Pharmacologically regulated Fas-mediated death of adoptively transferred T cells in a nonhuman primate model. *submitted for publication.*

30. Richard RE, Weinreich M, Chang K-H, Ieremia J, Stevenson MM, Blau CA: Modulating erythrocyte mixed chimerism in a mouse model of pyruvate kinase deficiency. *Submitted for publication.*

31. Masuko M, Weinreich M, Chien S, and Blau CA: Longterm Monitoring of Mpl-Based In Vivo Selection Using Chemical Inducers of Dimerization *In preparation.*

32. Zhao S, Weinreich M, Blau CA: In vivo selection using a JAK2-based cell growth switch. *submitted.*

Book Chapters

1. Blau CA and Stamatoyannopoulos G: Evolving therapies in globin chain disorders. In: Lechner K and Gadner H (eds): Haematology Trends '93, Schattauer Verlag, 1993.

2. Blau CA and Stamatoyannopoulos G: Regulation of fetal hemoglobin. In: Embury S, Hebbel R, Mohandas N, and Steinberg M (eds): Sickle Cell Disease. Raven Press, pp. 247-277, 1994.

3. Liakopoulou E, Blau CA, Stamatoyannopoulos G: Induction of fetal hemoglobin by short chain fatty acids. In: Hemoglobin Switching. Intercept Ltd, 333-338, 1995.

4. Blau CA: Current status of stem cell therapy and prospects for gene therapy. In: Rodgers G (ed): Sickle Cell Disease and Thalassemia. Baillieres Clinical Haematology, 11(1): 257-275, 1998.

5. Blau CA: In vivo selection of genetically modified bone marrow cells. In: Bertino JR (ed): Marrow protection: transduction of hematopoietic cells with drug resistance genes. In: Progress in Experimental Tumor Research. Karger Press, 36:162-171, 1999.

6. Blau CA: Selection of primary hemopoietic cells using chemical inducers of dimerization. In: Ikada Y, Shimizu Y (ed): Tissue Engineering for Therapeutic Use. Elsevier Science, 4:113-120, 2000.

Reviews

1. Blau CA and Stamatoyannopoulos G: Hemoglobin Switching and its Clinical Implications. **Current Opinion in Hematology** 1:136, 1994.

2. Blau CA and Stamatoyannopoulos G: Preemptive therapy for genetic disease. **Nature Medicine** 2:161-2, 1996.

3. Neff T and Blau CA: Pharmacologically regulated cell therapy. **Blood** 97:2535-2540, 2001.

Other Publications

1. Blau CA: Therapy for globin chain disorders (letter). **N Engl J Med** 329:364,1993.

2. Blau CA: Induction of fetal hemoglobin with erythropoietin (letter). **Nephron** 65:336, 1993.

3. Blau CA: Adverse effects of G-CSF in sickle cell syndromes – Inside Blood, **Blood** 97:3681, 2001.

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AUGMENTED CV

Carl Anthony Blau

TEACHING

1. I headed the red cell section of the hematology course for second year medical students and taught one of the small groups.

2. Research trainees:
   Tobias Neff, MD: 1/95-12/96, 6/00–4/02.
   Liqing Jin, MD - Hematologist, Beijing: 5/96 – 10/99
   Noppadol Siritanaratkul, MD - Hematologist, Thailand; 1/97 – 12/98.
   Robert E. Richard, MD, Ph.D. – Assistant Professor, Hematology, UW. 7/97- 1/03.
   Hui Zeng, MD – Hematologist, Beijing: 3/98 – 2/00.
   Shengming Zhao MD – Hematologist, Beijing, 5/99 – 12/02.
   Masayoshi Masuko MD – Hematologist, Japan, 10/99 – 4/02.
   Irina Kirillova, MD, Ph.D. Research in liver diseases. 8/00 – 7/03.
   Horst von Recum, PhD–Embryonic stem cells, 10/01-present.
   Wenjin Guo PhD-alternative dimerization systems. 2/02-present.
   Kenji Ihara, MD, Assistant Professor, Kyushu University, Japan, 7/02-present
   Yasuo Nagasawa, Ph.D., Lead Researcher, Sankyo, Tokyo, Japan, 10/02 - present
   Lazar Dimitrov, Graduate Student, Genome Sciences 7/03 – present
   Angelo DeClaro, MD, Hematology Fellow 9/03 – present
3. I organize the monthly meeting of the Seattle Gene Therapy Club, and the biweekly meeting of Stem Cell Club.

RESEARCH IN PROGRESS

*In vivo selection using a cell growth switch (*Jin et al., **Nature Genetics,** 26:64-6, 2000; Neff et al., **Blood**, 100:2026-2031, 2002, Zhao et al., in preparation).
Selection allows rare cells with a desired phenotype to emerge from a background of unwanted cells. Selection of cells within a living organism, termed *in vivo* selection, has the potential to overcome many of the current obstacles to gene therapy. Strategies for achieving *in vivo* selection have relied on genes that confer resistance to subsequently administered cytotoxic drugs, however these approaches entail toxicity to the organism as a whole. We have developed an alternative system for *in vivo* selection that uses a "cell growth switch," allowing a minor population of genetically modified cells to be directly, inducibly and specifically amplified, thereby averting the risks associated with cytotoxic drugs. This system provides a general platform for conditionally expanding genetically modified cell populations *in vivo* that may have widespread applications in gene and cell therapy. We have shown that this approach works in mouse and canine models. Studies in the primate model are ongoing. More recently, we have used the same approach to develop a JAK2-based cell growth switch.

Defining the signals that specify stem cell self-renewal (Zeng et al., **Blood** 98:328-334, 2001; Zhao et al., **EMBO J**, 21:2159-2167, 2002).
Defining signals that can support the self-renewal of multipotential hemopoietic progenitor cells (MHPCs) is pertinent to understanding leukemogenesis and may be relevant to developing stem cell-based therapies. We have defined a set of signals, JAK2 plus either c-kit or flt-3, which together can support extensive MHPC self-renewal. Phenotypically and functionally distinct populations of MHPCs were obtained, depending on which receptor tyrosine kinase, c-kit or flt-3, was activated.

Self-renewal was abrogated in the absence of STAT5a/b, and in the presence of inhibitors targeting either the mitogen activated protein kinase (MAPK) or phosphotidylinositol 3' kinase (PI-3K) pathways. These findings suggest that a simple two-component signal can drive MHPC self-renewal.

Selective expansion of genetically modified primary human hemopoietic cells using chemical inducers of dimerization (Richard et al., **Blood** 95:430-6, 2000, Richard et al., **Stem Cells 21:71-78, 2003**).
We've extended our studies using the dimerizer system to the selective expansion of transduced CD34+ cells of human cord blood origin. Transduced cells expanded an average of 186 fold in the presence of dimerizer. The responsive cell type was primarily erythroid. In more recent studies we have extended these observations to CD34+ cells from adult marrow. Furthermore, we have found that the cell lineages capable of responding to dimerizer can be modulated through the addition of growth factors. This work was described in an article published in the April 21, 2000 issue of the *Wall Street Journal* (page B6).

The first clinical gene therapy trial using chemical inducers of dimerization. In conjunction with Stan Riddell and our collaborators at Ariad Pharmaceuticals we are in the process of preparing to perform a clinical gene therapy trial in relapsed leukemia. Donor lymphocyte-mediated anti-tumor effects represent the single most important therapeutic benefit of allogeneic bone marrow transplantation (BMT). Paradoxically, donor lymphocyte-mediated Graft versus Host Disease (GVHD) represents the single greatest toxicity of allogeneic BMT. Over the past decade a large body of research has focused on harnessing the therapeutic potential of donor lymphocyte infusions while avoiding the development of life threatening GVHD. This proposal describes a pilot study that will test a new system that allows the survival of infused donor lymphocytes to come under pharmacological control. Donor lymphocytes are equipped with a suicide gene. In the past, HSV thymidine kinase has been used for this purpose, however the immunogenic nature of the HSV-TK protein will severely impede the use of this gene in future gene therapy trials. In order to reduce the likelihood of immunogenicity, it would be highly desirable to employ a suicide gene encoding a protein that is completely human in origin. Our collaborators at ARIAD Pharmaceuticals have developed such a system based on the human cell surface receptor Fas, which naturally signals apoptosis (programmed cell death) in T lymphocytes. Fas signaling is normally initiated by clustering of the receptor by its ligand, leading to a cascade of cytotoxic events. In the ARIAD system, clustering of an artificial Fas receptor (introduced by gene transfer) and consequent cell death is brought under the control of a small molecule drug Binding of this "dimerizer" clusters the chimeric Fas receptors and initiates the natural apoptotic cascade. All the protein components of this system are human in origin, therefore the peptide sequences at the fusion sites and a point mutation in FKBP12 represent the only potentially immunogenic sequences. 30 patients receiving donor lymphocyte infusions for relapse of hematological malignancy will be enrolled. We plan to begin enrollment into this trial in September 2002.

Other Applications of the Dimerizer System
We have embarked on a series of collaborative studies to determine the utility of using chemical inducers of dimerization to stimulate expansion of genetically modified liver cells (with Andre Lieber), pancreatic beta cells (with Ake Lernmark and Andre Lieber), and muscle cells (with Charles Murry). If successful, these approaches might prove useful for treating liver diseases, diabetes, and other disorders.

Preliminary Studies for Gene Therapy in Sickle Cell Disease and β Thalassemia

In order to perform gene therapy for these disorders, we will need to procure stem cells. The most widely used method for obtaining stem cells is to promote their mobilization into the peripheral blood using the cytokine GCSF so that they can then be collected by leukapheresis. We have previously shown that GCSF can produce life-threatening complications in patients with sickle cell disease (*Lancet*, 1998).

Our experience with GCSF strongly suggests that alternative means will need to be found for the procurement of stem cells in patients with sickle cell disease. A protocol to study the use of hydroxyurea for the mobilization of progenitors in patients with sickle cell anemia has been approved by the GCRC's Scientific Advisory Committee and the UW Human Subjects Committee, and has enrolled three patients. In collaboration with Bob Richard and Erica Jonlin, we are also examining the safety of stem cell collection in patients with sickle cell anemia. This work is part of the Program for Excellence in Gene Therapy.

Development of a Clinical Gene Therapy Program at the University of Washington
As Associate Program Director for Gene and Cell Therapy at the GCRC I have taken part in the effort to establish a world class gene therapy program at UW. Over the last 2 years I worked with Dr. Oliver Press to establish his gene therapy program on the CRC. I am also Director of the Clinical Core for the Program for Excellence in Gene Therapy, and am building the infrastructure for clinical gene therapy trials at UW.

PATIENT CARE ACTIVITIES

1. I attend on the Hematology Consultation Service for one to two months per year, and on the stem cell transplant service or general oncology another month per year.

2. I maintain a relatively busy Hematology Clinic one half day per week, with emphasis on patients with sickle cell anemia and β thalassemia.

SCIENCE

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Rational Design of Potent Antagonists to the Human Growth Hormone Receptor

Germaine Fuh, Brian C. Cunningham, Rikiro Fukunaga, Shigekazu Nagata, David V. Goeddel, and James A. Wells*

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Rational Design of Potent Antagonists to the Human Growth Hormone Receptor

Germaine Fuh, Brian C. Cunningham, Rikiro Fukunaga, Shigekazu Nagata, David V. Goeddel, James A. Wells*

A hybrid receptor was constructed that contained the extracellular binding domain of the human growth hormone (hGH) receptor linked to the transmembrane and intracellular domains of the murine granulocyte colony-stimulating factor receptor. Addition of hGH to a myeloid leukemia cell line (FDC-P1) that expressed the hybrid receptor caused proliferation of these cells. The mechanism for signal transduction of the hybrid receptor required dimerization because monoclonal antibodies to the hGH receptor were agonists whereas their monovalent fragments were not. Receptor dimerization occurs sequentially—a receptor binds to site 1 on hGH, and then a second receptor molecule binds to site 2 on hGH. On the basis of this sequential mechanism, which may occur in many other cytokine receptors, inactive hGH analogs were designed that were potent antagonists to hGH-induced cell proliferation. Such antagonists could be useful for treating clinical conditions of hGH excess, such as acromegaly.

Knowledge of the molecular basis for hormone action is key to the rational design of hormone agonists and antagonists. High-resolution mutational analysis (*1*, *2*) and x-ray crystallographic studies (*3*) have defined two sites on hGH for binding two molecules of the extracellular domain of its receptor (hGHbp) (*4*). Dimerization of the hGHbp occurs sequentially, such that a hGHbp molecule binds to site 1 and then a second hGHbp molecule binds to both site 2 on hGH and a site on the first hGHbp (Fig. 1). A thorough examination of the biological importance of this model has been precluded because of the lack of an adequate cellular signaling assay for hGH. Here, we constructed a sensitive, cell-based assay for hGH, investigated the mechanism for signal transduction, and applied the assay for the design of antagonists to the hGH receptor.

The hGH receptor belongs to a large family of receptors of hematopoietic origin (*5*) that includes the interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) receptors. An IL-3–dependent myeloid leukemia cell line (FDC-P1) transfected with the full-length murine G-CSF (mG-CSF) receptor is stimulated to proliferate by G-CSF without IL-3 (*6*). We constructed a hybrid receptor that contained the hGHbp linked to a portion of the mG-CSF receptor containing the three extracellular fibronectin repeats and the transmembrane and intracellular domains (*7*). The fibronectin domains do not participate in the binding of G-CSF but are required for efficient expression of the mG-CSF receptor (*6*).

Competitive displacement of ^{125}I-labeled hGH from hybrid receptors on whole cells was used to establish the affinity for hGH and the approximate number of receptors per cell (*8*). In several independent

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binding experiments, the apparent dissociation constant (K_d) value for hGH was 0.1 ± 0.03 nM, and there were 1000 ± 300 receptors per cell. This affinity is about three to four times stronger than that for hGH binding to the soluble hGHbp and may reflect a high local concentration of receptors on cells (an avidity effect). Nontransfected cells lacked specific binding sites for hGH (9). At low concentrations, hGH induces cell proliferation with a median effective concentration (EC_{50}) of ~20 pM (Fig. 2A), a value somewhat lower than the apparent K_d for binding to whole cells (~100 pM). This may indicate that signaling for maximal cell proliferation requires less than total receptor occupancy.

Each hGH molecule is bivalent because it contains two separate sites for binding the hGHbp (Fig. 1). In contrast, the hGHbp is effectively univalent because each site uses virtually the same determinants to bind to either site 1 or site 2 on hGH (3). Excess hGH will dissociate the hGH·$(hGHbp)_2$ complex to form a hGH·hGHbp complex in which hGH is bound exclusively at site 1 to the hGHbp (1). Thus, excess hGH should antagonize signaling by preventing dimerization (Fig. 1). Indeed, at very high hGH concentrations the proliferation activity is lost [concentration required to inhibit proliferation by 50% (IC_{50}) ≅ 2 μM]. Cell proliferation induced by IL-3 was not altered in the presence of high concentrations of hGH (8 μM); thus, 8 μM hGH is not toxic to cells (9). This effect appear not to involve cross-linking of receptor between cells or other cell-to-cell interac tions because the effects of hGH were nc influenced by cell density. Furthermore, th assay is specific because FDC-P1 cells tha contain the full-length mG-CSF recepto do not respond to hGH and cells tha contain the hybrid receptor do not respor to G-CSF (10).

To further investigate the requireme

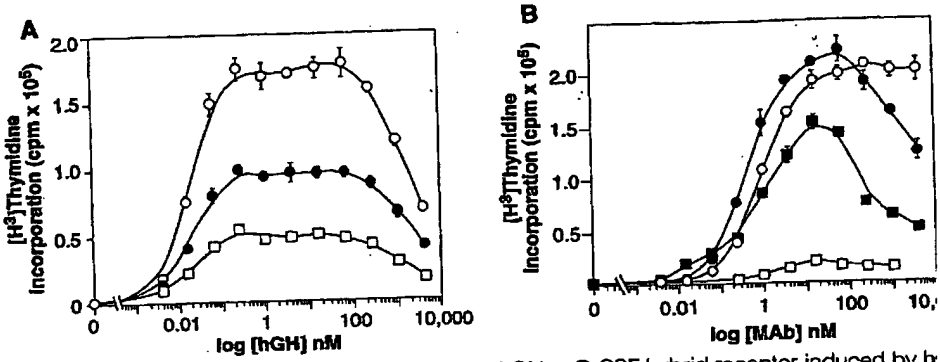

Fig. 2. Proliferation of FDC-P1 cells containing the hGH–mG-CSF hybrid receptor induced by h (7) (**A**) or induced by MAbs to the hGH receptor (**B**) (17). In (A), cells were grown in RPMI 1(media supplemented with IL-3 (10 U/ml), 10 μM β-mercaptoethanol, and 10% FBS at 37°C and CO_2 (6). Cells were washed with the same medium without IL-3. Cells were added to 96-well pla at a density of 4 × 10^5 cells per milliliter (○), 2 × 10^5 cells per milliliter (●), and 1 × 10^5 cells milliliter (□) in 100 μl. Cells were then treated with various concentrations of hGH for 18 hours. measure DNA synthesis, we added ^{3}H-labeled thymidine (1 μCi per well) to each well. After 4 ho cells were collected and washed on glass filters. Scintillation cocktail (2 ml) was added, radioactivity was counted with a Beckman LS1701 scintillation counter. In (B), cells were cultu as in (A) and plated at a density of 4 × 10^5 cells per milliliter in medium containing vari concentrations of anti-hGH receptor MAb 263 (●), MAb 13E1 (○), MAb 3D9 (■), or MAb 5 (□). A 18 hours at 37°C, cells were washed, and the amount of [^{3}H]DNA synthesized was determinec scintillation counting as in (A). Each data point represents the mean of triplicate determinations, error bars indicate the SD.

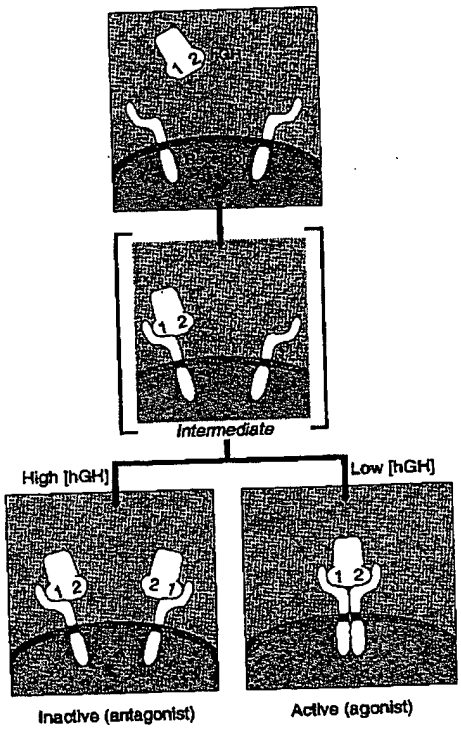

Fig. 1. Sequential dimerization model for activation of the hGH–mG-CSF hybrid receptor. At low concentrations, hGH binds first at site 1 and subsequently at site 2 (as indicated) to produce an active hGH·$(hGHbp)_2$ complex. At high concentrations, hGH saturates the receptor through site 1 interactions and acts as an antagonist. We show the receptors dissociated initially because, in the absence of hGH, the hGHbp does not self-dimerize as shown by ultracentrifugation for concentrations <0.1 mM. Nonetheless, it is possible that some full-length receptors are loosely pre-dimerized and become activated upon sequential binding of hGH.

Table 1. Summary of dose-response data for a variety of anti-hGH receptor MAbs, FAbs (17), hGH mutants (12) for stimulating proliferation of FDC-P1 cells containing the hGH–mG-CSF hy receptor. "None" indicates that no effect was observed; ND, not determined. K_d values for M binding to the hGHbp were taken from (14). The K_d values for hGH and variants were measured a competitive displacement assay in which ^{125}I-labeled hormone bound to hGHbp was precipit with MAb 5 (2, 13). This gives the affinity for the monomeric hGH·hGHbp complex. Values for I were taken from titration curves shown in Fig. 2, A and B, and Fig. 4 and represent the half-max concentration for stimulation of cell proliferation. Data are the mean of triplicate assays, and the were within 15% of mean. Values shown with > indicate that maximal stimulation or inhibitic proliferation was not detected at the concentrations tested. For these cases, we report estimates of the EC_{50}. IC_{50} refers to the concentration leading to 50% inhibition of maxima proliferation.

Protein	K_d (nM)	EC_{50}	IC_{50} (self-antagor
MAb 263	0.6	0.3 nM	~3 μM
MAb 13E1	3.2	0.8 nM	>10 μM
MAb 3D9	2.2	0.8 nM	0.2 μl
MAb 5	0.7	~2.5 nM	>1 μM
FAb 263	ND	>1.5 μM	ND
FAb 13E1	ND	>3 μM	ND
FAb 3D9	ND	>0.1 μM	ND
FAb 5	ND	>1 μM	ND
hGH	0.3	20 pM	2 μM
K172A/F176A	200	25 nM	None
G120R	0.3	None	None
H21A/R64K/E174A	0.01	20 pM	60 nM
H21A/R64K/E174A/G120R	0.01	None	None

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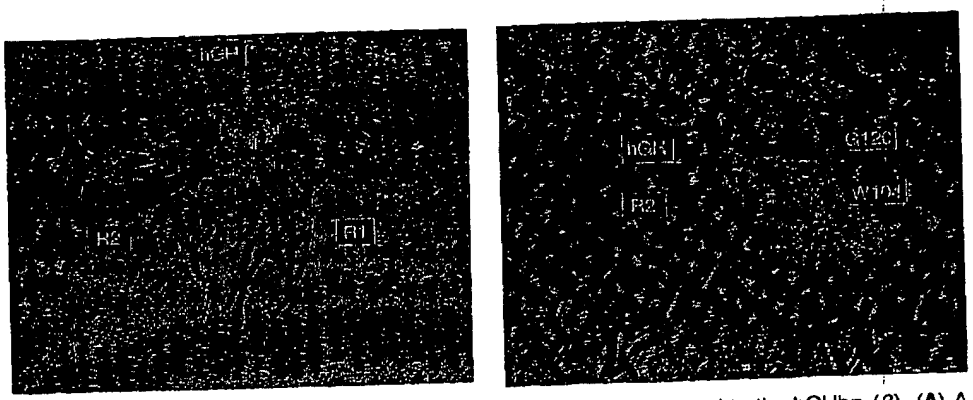

Fig. 3. Molecular models based on x-ray crystallography of hGH bound to the hGHbp (*3*). (**A**) A ribbon diagram of hGH (white) bound to two molecules of the extracellular domain of the hGH receptor (hGHbp; gray and black). The α carbon positions of mutant residues in hGH are shown by black dots. K172 and F176 are located in site 1 (interface with black hGHbp) and G120 is located in site 2 (interface with gray hGHbp). Gray dots indicate structures in the hGHbp that are not well defined by the electron density. The model is based on a 2.7 Å resolution x-ray structure of the complex (*3*). (**B**) A close-up showing that G120 located on helix 3 of hGH makes van der Waals contact with W104 from the hGHbp bound to site 2. R1 and R2, receptor 1 and receptor 2, respectively.

for dimerization of the hGHbp to signal in the hybrid receptor cell proliferation assay, we used bivalent monoclonal antibodies (MAbs) and univalent fragments derived from them (FAbs) that recognized the hGHbp. At low concentrations, three of four different MAbs to the receptor were as potent as hGH in inducing cell proliferation (Fig. 2B and Table 1). The EC_{50} value for each MAb (0.3 to 1 nM) was usually somewhat less than the K_d value determined by enzyme-linked immunosorbent assay (Table 1). As with hGH, this may reflect avidity effects on whole cells or that maximal signaling is achieved at less than 100% receptor occupancy, or both. At much higher concentrations (0.2 to ~3 μM), two of these MAbs were less effective at stimulating proliferation, presumably because excess MAb blocks receptor cross-linking by binding monovalently to hGHbp. Corresponding monovalent FAb fragments had little or no effect on cell proliferation (Table 1), which indicates further that bivalency is required for signaling activity.

The differences in stimulation of cell proliferation at low concentrations and inhibition at high concentrations for these MAbs (Fig. 2B) can be explained by the different ways they bind to the hGHbp. MAb 5 prevents binding of a second hGHbp to the hGH·hGHbp complex (*1*), possibly by binding to the region where both receptors contact each other (Fig. 1). The fact that MAb 5 is the least efficient at stimulating proliferation may indicate that the receptors need to approach each other closely for optimal signaling. MAb 13E1 did not inhibit proliferation at the concentrations tested. This MAb blocks hGH binding (*11*) and probably binds like hGH to form very stable receptor dimers. In contrast, MAbs 263 and 3D9 bind at sites away from the hormone-receptor interfaces (*11*) and show similar agonistic and antagonistic effects on proliferation. Maximal stimulation of proliferation by hGH occurred over a wider range of concentrations than did maximal stimulation by MAbs 263 and 3D9, perhaps because with hGH bound, the dimers have the optimal receptor-to-receptor contacts. The fact that MAbs 263 and 3D9 are agonists suggests that the structural constraints for formation of active dimers are rather loose.

FAb fragments derived from MAb 13E1 or MAb 5 antagonized hGH-induced cell proliferation, whereas those derived from MAbs 263 and 3D9 did not (Table 2). These studies are consistent with the fact that the binding of MAb 13E1 or MAb 5 to their epitopes blocks hormone-to-receptor or receptor-to-receptor interfaces, respectively.

Table 2. Summary of antagonist effects of FAb and hGH analogs that block hGH-induced ce proliferation of FDC-P1 cells containing th hybrid hGH–mG-CSF receptor. Cells were i cubated with 1 nM hGH and various concentr tions of FAb (*17*) or hGH analog (*12*). The IC_{50} the concentration required to block 50% of t cell proliferation activity of hGH. "None" inc cates that no inhibition was observed at conce trations of FAb or hGH analog of up to 10 μM

Protein	IC_{50}
FAb 263	None
FAb 13E1	0.8 μ
FAb 5	0.2 μ
FAb 3D9	None
hGH	2 μM
K172A/F176A	None
G120R	20 nM
H21A/R64K/E174A	60 nM
H21A/R64K/E174A/G120R	2 nM

To determine the structural requi ments for dimerization of hGH (Fig. 1), examined mutants of hGH that were signed to reduce binding of the receptor site 1 or site 2 (Fig. 3). The mutant K172 F176A (*12*), which preserves site 2 det minants but alters important side chains site 1, promoted cell proliferation, but EC_{50} was shifted to a concentration ab 10^3 times higher than that of wild-t hGH (Fig. 4 and Table 1). This is con tent with the 560-fold reduction in affinity for site 1 binding of the K17 F176A mutant as compared to that of wild-type hGH when measured in v (*13*). No inhibition of proliferation w K172A/F176A was observed at the conc trations tested.

On the basis of the x-ray structure of hGH·$(hGHbp)_2$ complex (*3*), we desig

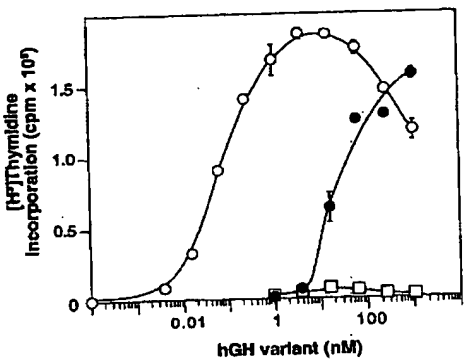

Fig. 4. Proliferation of FDC-P1 cells containing the hGH–mG-CSF hybrid receptor, caused by increasing concentrations of wild-type hGH (○), the site 1 hGH variant K172A/F176A (●), and the site 2 hGH variant G120R (□). Cells were cultured, treated, and assayed as described in Fig. 2A, except that cells were treated for 18 hours with [³H]thymidine. The hGH mutants were prepared and purified as described (*2, 12*).

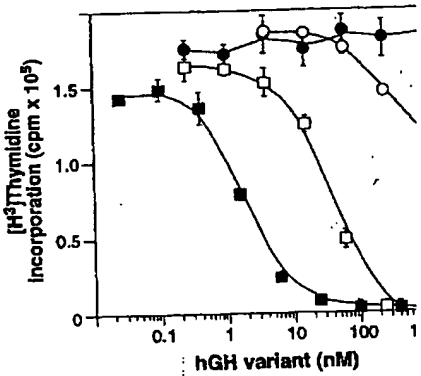

Fig. 5. Antagonism of hGH-induced cell p eration by hGH variants. Cells were prep as in Fig. 2A and incubated with 1 nM hGH various concentrations of the site 1 m K172A/F16A (●), the site 2 mutant G120R the combined enhanced site 1 and site 2 tant (H21A/R64K/E174A/G120R) (■), wild-type hGH (○).

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a mutant G120R, which retains a functional site 1 but on which site 2 is sterically blocked (Fig. 3B). This variant did not affect cell proliferation at the concentrations tested (Fig. 4). Thus, binding to either site 1 or site 2 is necessary but not sufficient for promoting cell proliferation.

If the sequential signaling mechanism (Fig. 1) is correct, mutants blocked in site 2 binding (but not in site 1 binding) should antagonize hGH-induced cell proliferation. To test this, we cultured cells with enough hGH (1 nM) to support 90% of maximal cell proliferation and added increasing concentrations of wild-type hGH or the mutants in site 1 (K172A/F176A) or site 2 (G120R). As expected, the site 2 mutant antagonized hGH whereas the site 1 mutant was ineffective (Fig. 5). In fact, the site 2 mutant was nearly 100 times more potent as an antagonist (IC_{50} = 20 nM) (Table 2) than wild-type hGH (IC_{50} = 2 μM). For hGH to be antagonistic, free hormone must react with free receptors before the hGH-bound intermediate does so. This only occurs at high concentrations of hGH. In contrast, once G120R is bound, it cannot dimerize and agonize the receptor. Thus, G120R as an antagonist does not need to compete against G120R as an agonist.

Although G120R is a much more potent antagonist than hGH, 20 nM G120R was required to inhibit by 50% the proliferative effect of 1 nM hGH (Table 2). This may reflect the fact that hGH is bound through interaction of sites 1 and 2 with two receptors more tightly than G120R is bound in the complex with a single receptor through site 1 alone. Furthermore, maximal signaling by hGH may not require 100% receptor occupancy. In either case, improving the affinity of site 1 for hGHbp in the G120R mutant should make it a more potent antagonist.

Single-site hGH variants have been produced (*2*, *14*) that bind more tightly to the hGHbp at site 1. A variant that contains all three of these mutations (H21A/R64K/E174A) bound 30 times more tightly than wild-type hGH to the hGHbp (Table 1). This variant had an IC_{50} for inhibiting proliferation that was about 30 times lower than that of hGH. This is consistent with the notion that the inhibitory effect results from competition for binding to hGHbp between site 2 on the bound hormone-receptor intermediate and the free site 1 on the soluble hormone. The fact that improvement in site 1 binding affinity did not improve the efficacy of the hormone as an agonist may be understood upon future analysis of the on and off rates.

We further mutated this variant by changing Gly^{120} to Arg. The mutant with all four modifications was ten times more potent than G120R as an hGH antagonist (Fig. 5 and Table 2). This is further evidence for the importance of site 1 binding properties for antagonism.

Our data suggest that the inhibition of proliferation caused by hGH, MAbs, and their derivatives is the result of blocking receptor dimerization rather than causing down-regulation of receptors. First, cells propagated with IL-3 instead of hGH do not show a greater hGH response or hGH receptor number (*9*). Second, receptor down-regulation is usually correlated to receptor activation. The ratio of EC_{50} to IC_{50} for each of the MAbs and hGH varies widely, which shows that receptor activation can be readily uncoupled from inhibition by the alteration of binding properties. Finally, the G120R mutant is inactive as an agonist, although it is a more potent antagonist than hGH (Fig. 5), and pretreatment of cells with G120R does not enhance its antagonistic effect (*9*). Thus, the antagonistic effect of G120R is not consistent with receptor down-regulation. It is possible that the inhibitory effects observed for other hormones at high concentrations may occur because receptor dimerization is blocked by self-competition.

Our studies indicate that sequential dimerization is crucial for hybrid-receptor activation. Knowledge of this mechanism and the structural (*3*) and functional (*1*, *2*) properties of the binding interfaces allowed us to design potent antagonists to the hGH receptors, which may be useful in the clinical treatment of hGH excess acromegaly (*15*). In fact, a transgenic strain of mice that expresses large amounts of bovine GH altered in site 2 produces dwarf mice (*16*). This mechanism-based strategy for design of potent antagonists for hGH may be applicable to other hormones such as prolactin, placental lactogen, IL-2, IL-3, IL-6, G-CSF, granulocyte-macrophage–CSF, erythropoietin, and related hematopoietines and cytokines (*5*) if sequential binding of two receptors to a single hormone molecule is required for their signaling.

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7. A hybrid receptor was constructed from cDNA that contained exons 1 through 7 of the hGH receptor (which encode the secretory signal and the extracellular hGH binding domains) linked to exons 9 through 17 of the mG-CSF receptor (which encode the three fibronectin domains and the entire transmembrane and intracellular domains). The protein encoded by the hybrid cDNA contains the amino acids −18 to 243 of the hGH receptor and 309 to 812 of the mG-CSF receptor. In this construction, Cys^{241} was replaced by Arg, which has no effect on binding to hGH [G. Fuh *et al.*, *J. Biol. Chem.* 265, 3111 (1990)]. Sequences derived from the hGH receptor [D. W. Leung *et al.*, *Nature* 330, 537 (1987)] and from the mG-CSF receptor [R. Fukunaga *et al.*, *Cell* 61, 341 (1990)] were cloned by means of the polymerase chain reaction [R. Higuchi, in *PCR Protocols: A Guide to Methods and Applications*, M. Innis *et al.*, Eds. (Academic Press, New York, 1989), pp. 177–183] into the vector pER-BOS (*6*). The hybrid cDNA was introduced into mouse FDC-P1 cells as described (*6*). Stable transformants that expressed the hybrid protein were identified by their ability to bind ^{125}I-hGH (*8*).
8. Cells grown with IL-3 were washed before the assay with phosphate-buffered saline (PBS) that contained 10% fetal bovine serum. Cells (1.2 × 10^6 per milliliter) were incubated with serial dilutions of hGH in the presence of ^{125}I-labeled hGH variant Y103A (20 pM) for 18 hours at 4°C. The Y103A variant was used to prevent iodination of Y103, which inhibits the binding of the receptor to site 2 (*9*). Cells were washed with PBS twice to remove the excess hormone, and the bound radioactivity was counted.
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12. Mutants of hGH were prepared by site-directed mutagenesis [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* 154, 367 (1987)] and purified as described (*2*, *13*). Mutants are designated by the wild-type residue followed by the position and the mutant residue in the single-letter code A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Try; and Y, Tyr. Thus, G120R indicates that Gly^{120} was converted to Arg. Multiple mutants are represented as a series of single mutants separated by slashes.
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17. The MAbs 5 and 263 were from Agen (Parsippany, NJ) and have been described [R. Barnard, P. G. Bundesen, D. B. Rylatt, M. J. Waters, *Endocrinology* 115, 1805 (1984); *Biochem. J.* 231, 1 (1985)]. The MAbs 13E1 and 3D9 were from Genentech hybridoma group, and their properties have been described (*1*, *11*). Briefly, MAbs were purified from mouse ascites fluid by binding to Protein A–Sepharose (Pharmacia) and eluted with 0.1 M acetate (pH 3.0). FAb fragments were prepared by treating MAbs with dithiothreitol activated papain (1 part to 50 parts MAb by weight) in PBS with 10 mM cysteine for 1 h. Digestions were stopped by adding 0.1 M iodoacetamide. The Fc and residual MAb were removed by adsorption onto Protein A–Sepharose and the FAb fragments were further purified by gel filtration on Superose 12 (Pharmacia).
18. We thank B. de Vos for providing x-ray coordinates for the hGH·$(hGHbp)_2$ complex and useful comments on the manuscript, E. Ishizaka-Ikeda for help in constructing the hybrid hGH-G-CSF receptor, T. Hynes for help with molecular modeling, the oligonucleotide synthesis group at Genentech for DNA synthesis, and M. Thorner and C. Silva for sharing data on hGH stimulation of IM-9 cells before publication.

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